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REVIEW

International Council for Standardization in Haematology (ICSH) recommendations for laboratory measurement of ADAMTS13

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Abstract

This guidance document was prepared on behalf of the International Council for Standardization in Haematology (ICSH), by the ADAMTS13 Assay Working Group, which comprises an international group of both clinical and laboratory experts. The document provides recommendations on best practice for the performance of ADAMTS13 assays in clinical laboratories. ADAMTS13 assays support the differential diagnosis of thrombotic microangiopathies and have utility in the management of thrombotic thrombocytopenic purpura (TTP). There are three types of assay: activity, antigen and autoantibody/inhibitor assays. Methods for activity assays differ in terms of sensitivity, specificity, precision and turnaround time. The most widely used assays involve VWF peptide substrates and either chromogenic ELISA or FRET techniques, although chemiluminescence assays and rapid screening tests have recently become available. Tests for autoantibodies and inhibitors allow confirmation of acquired, immune-mediated TTP, while antigen assays may be useful in congenital TTP and as prognostic markers. In this document, we have attempted to describe ADAMTS13 assays and the conditions that affect them, as well as: blood collection, sample processing, quality control, standardization and clinical utility; recognizing that laboratories in different parts of the world have varying levels of sophistication. The recommendations are based on expert opinion, published literature and good clinical laboratory practice.

KEYWORDS

ADAMTS13, haemostasis, thrombosis, VWF

1 | BACKGROUND

ADAMTS13 (A disintegrin and metalloprotease with a thrombospondin type 1 motif, member 13) is a plasma metalloprotease which is critically involved in the control of von Willebrand Factor (VWF), its major substrate.[1] Secretion from the hepatic stellate cell is thought to be the main plasma source of ADAMTS13, although small quantities also appear to be produced by the vascular endothelium, podocytes in the kidney glomeruli and it may be detectable in platelets.[2] VWF is released as an ultralarge molecular weight

multimeric form (ULVWF), which is rapidly cleaved by ADAMTS13, resulting in progressively smaller multimers.[1] The high shear stress found in small arterioles unravels the globular form of ULVWF so that the metalloprotease domain of ADAMTS13 can come into close proximity with the cleavage site in the VWF A2 domain, allowing cleavage. The other domains of ADAMTS13 play a role in the attachment and unravelling of VWF, while VWF itself induces conformational changes in ADAMTS13, making it fully active.[3-7]

Thrombotic microangiopathies (TMA) are a group of disorders characterized by thrombocytopenia and microangiopathic haemolytic anaemia (MAHA) resulting in varying degrees of organ damage; they are rare, but potentially life-threatening unless they are recognized and treated rapidly. They include thrombotic thrombocytopenic purpura (TTP) and haemolytic uraemic syndrome (HUS),[8,9] but may also be secondary to cancer, viral infection (eg HIV), organ or bone marrow transplantation, pregnancy (preeclampsia/eclampsia or HELLP syndrome), severe hypertension, drugs, autoimmune disorders, sepsis and disseminated intravascular coagulation.[10-12] In thrombotic thrombocytopenic purpura (TTP), there is a loss of ADAMTS13 function resulting in the prolonged circulation of ULVWF, which has an increased platelet binding capacity and may participate in platelet aggregate formation in the microcirculation with sequestration and consequent thrombocytopenia. Blood flow is limited, causing ischaemic organ damage, and haemolysis occurs due to red cell mechanical destruction. Elevated levels of indirect bilirubin and lactate dehydrogenase are common due to the gross haemolysis. Diagnosis is based on the presence of thrombocytopenia and MAHA, as well as the clinical history and examination of the blood film[13]; additional laboratory studies are required to distinguish between different causes of TMA.

TTP exists in acquired, immune-mediated (iTTP) and congenital (cTTP) forms.[14] iTTP is caused by the presence of autoantibodies to ADAMTS13, which decrease its function or increase ADAMTS13 clearance from the circulation. These antibodies are heterogeneous, varying in epitope specificity and avidity. iTTP can occur at any age, mostly in the 3rd-5th decade of life, and is frequently associated with infection and pregnancy, but may also occur secondary to a variety of other conditions. Patients with iTTP who enter remission may relapse at varying intervals, most recurrences occurring during the first year due to the reappearance of the autoantibody, loss of ADAMTS13 and development of ULVWF. In cTTP, which usually presents in childhood, the loss of ADAMTS13 activity is caused by pathogenic genetic *ADAMTS13* variants, resulting in decreased synthesis, secretion, or function.

Later onset cTTP may also occur, often during pregnancy. ADAMTS13 assays are useful to distinguish TTP from other TMA and, in the untreated patient at presentation, a severe deficiency (<10 IU/dL) of ADAMTS13 activity is diagnostic for TTP.[14] ADAMTS13 activity has been listed as a critical test.[15] Plasma levels and function of ADAMTS13 are modulated by hepatic stellate cell damage, cytokine levels and oxidation (which inhibits ADAMTS13 proteolytic activity and makes VWF resistant to cleavage)[2]; a mild to moderate reduction in activity may occur in cardiovascular disease, liver disease, sepsis and consumptive coagulopathies, with. Guidance on the diagnosis of TTP, management, therapy and prophylaxis are available[13,16,17] and ICSH has published recommendations on schistocyte enumeration in TMA.[18]

HUS[9] may be subdivided into infection-associated HUS (IA-HUS) and complement-mediated HUS (CM-HUS, formerly termed atypical HUS). IA-HUS is typically associated with Shiga toxin-induced bloody diarrhoea in children, which is treated with supportive care, and in some cases renal dialysis. CM-HUS, more common in adults, usually results from defective regulation of the complement system and may sometimes show multisystem symptoms similar to TTP, making the differential diagnosis difficult. Unlike TTP, ADAMTS13 levels are usually normal in CM-HUS, although they sometimes show a moderate reduction (30-50 IU/dL). ADAMTS13 assays are thus pivotal in differentiating TTP and CM-HUS, which require different treatments. Plasma exchange (PEX) and immunosuppressive agents are typically used for TTP[8,13] while PEX and complement inhibition (eg with eculizumab) are used in CM-HUS,[19] although PEX may be used until a firm diagnosis is made.

The aim of this ICSH document is to provide laboratory guidance and an up-to-date summary of best practice for the validation, performance, and reporting of ADAMTS13 assays. The consensus recommendations provided are based on information from peer-review publications, the authors' personal experience and expert opinion, as well as good clinical laboratory practice.

2 | PRE-ANALYTICAL VARIABLES

Citrated (0.109 mol/L sodium citrate) platelet-poor plasma is normally used for ADAMTS13 assays. Samples should be prepared to ensure platelet depletion ($<10 \times 10^9$/L; ie centrifugation at 2000 *g* for 15 minutes). This should be performed at room temperature (18-20°C), particularly if other haemostasis assays might be performed, to avoid potential platelet changes and proteolytic activation. Serum and heparinized plasma have been used and may be advantageous in certain types of assay, where high sensitivity is required, with less sample dilution, and where citrate chelation of divalent cations interferes with the assay. However, laboratories must validate blood collection into alternative anticoagulants before clinical application. Plasma or serum samples should be rapidly removed from the cells using a plastic transfer pipette, avoiding disturbance to the buffy coat and stored in appropriate polypropylene containers.

ADAMTS13 is present in serum, but inaccurate activity results could potentially occur, due to platelet and leucocyte activation as well as ADAMTS13 degradation, by thrombin and other proteases. However, this can be effectively mitigated with protease inhibitors. It should be noted that ADAMTS13 activity can also vary significantly due to other factors, for example changes in assay buffer conditions (pH, ionic strength and surfactants) can affect ADAMTS13 activity due to allosteric mechanisms.[3-6] EDTA plasma cannot be used for activity assays, as it irreversibly disrupts the quaternary

structure of ADAMTS13, causing false low/absent activity. In cases of unexpected absence of ADAMTS13 activity, where results do not fit the clinical picture and to exclude pre-analytical variables such as incorrect sample type, a prothrombin time or activated partial thromboplastin time on the sample can be helpful as grossly prolonged times suggest EDTA contamination or serum. Alternatively, as EDTA samples have markedly elevated potassium and decreased calcium,[20] electrolyte analysis showing an abnormal sodium to potassium ratio suggests EDTA anticoagulant.

Blood samples should be collected with minimal stasis and processed rapidly to avoid cellular and plasma activation. Any significant delays in processing should be recorded and depending on the laboratory policy, added as a comment to the report. Clotted samples or those containing clumped platelets are not suitable. Samples that are not being assayed on the same day should be stored frozen at below −40°C.

If the samples are shipped to another site, dry ice should be used to avoid slow thawing during transit and comparable results can then be achieved between different laboratories.[21] Although ADAMTS13 has been shown to have good stability at room temperature,[22] some of these studies have employed samples from healthy normal subjects and it is not clear whether stability is good in all clinical samples, particularly those with critical illness and the potential for reduced plasma protease inhibitor levels. Before assay, frozen samples should be warmed to 37°C in a water bath for 5-10 minutes, until completely thawed and mixed gently to ensure that any cryoprecipitate is dissolved. Thawing for longer than this is undesirable as ADAMTS13 activity is lost after prolonged periods at 37°C.[23]

Icteric samples may not be suitable for some assay types (see specificity section below) due to interference in absorbance measurement or quenching of fluorescence signal.

Haemolysis is frequently seen in patients with TMA, due to entrapment and lysis of red cells in the microcirculation. However, where it is due to in vitro haemolysis, for example caused by a slow blood draw, fresh samples should be obtained. Variable ADAMTS13 activity may also be obtained in samples with gross lipaemia, although the exact nature of any influence of plasma lipids on activity assays has not been investigated and might possibly vary depending on the composition of the lipid.

Samples for TTP diagnosis are optimally collected prior to initiation of PEX, although samples collected early in treatment may still be informative. It is important that the laboratory has details of the type and timing of treatment.

2.1 | Consensus recommendations on sample collection and handling for ADAMTS13 assays

- Citrated plasma (centrifuged to ensure platelet depletion) should normally be used.
- Heparin plasma or serum samples may be used depending on the assay type and if it is validated for these sample types.
- EDTA plasma is not suitable for ADAMTS13 assays.
- Samples should be centrifuged and plasma separated from the cells as rapidly as possible after blood collection to avoid in vitro changes.
- Unless assays are performed immediately, plasma samples should be stored and shipped below-40°C to avoid potential proteolysis.

3 | ADAMTS13 ASSAYS

There are a variety of in-house and commercial methods for ADAMTS13 activity, antigen, inhibitors and ADAMTS13 antibody assay.

3.1 | Activity assays

The widespread use of ADAMTS13 assays has been limited by the rarity of TTP in the general population, technical difficulty of the assays and the length of time taken to generate an assay result.[24] First generation activity assays involved incubation of patient plasma with VWF and measurement of residual VWF by multimer electrophoresis,[25] SDS PAGE and Western Blotting with VWF antibodies,[26] Immunoradiometric assay,[27] or ristocetin cofactor assay,[28] or collagen binding ELISA (CBA).[29]

Electrophoretic detection of VWF multimers and analysis by ristocetin cofactor assay are difficult, with poor precision. Assays based on residual VWF binding to collagen are also time consuming, but the ELISA principle allows higher throughput, with better sensitivity and precision. However, there are numerous potential sources of error, including the use of denaturing agents that besides unravelling VWF could alter ADAMTS13 structure and dissociate ADAMTS13 autoantibodies.

Second generation activity assays employ peptide substrates based on the ADAMTS13 cleavage site in the VWF A2 domain. These are rapid, producing results within a few hours, with high throughput and good precision. However, these assays may fail to detect certain inherited ADAMTS13 defects, as they measure metalloprotease activity independent of exosite interactions with other VWF domains. Similar problems can occur where ADAMTS13 autoantibodies against these exosites exist. Elevated endogenous VWF levels influence some assays, and increased bilirubin levels can interfere in fluorescence-based techniques. Some of the current activity assays show relatively poor sensitivity, which can be an issue in the clinical management of TTP. The key principles of some of the activity assays have been reviewed[20]; they may be divided into assays utilizing full-length VWF as substrate and those using peptides or fragments derived from VWF. The details of the more popular assays are given in Table 1.

The most commonly used peptide substrate assays employ a 73 amino acid peptide (VWF73), based on the sequence around the cleavage site in the VWF A2 domain,[30] although some methods use

TABLE 1 Some popular clinical laboratory methods for ADAMTS13 activity

Method	Performance	Calibrant	Diluent; dilution factor	VWF substrate type	Detection; assay type
[a]CBA[29,48,60,76]	LLOQ = 5%-6% Intra-assay CV = 9% Inter-assay CV < 15%	PNP diluted in assay buffer	5 mmol/L Tris/BSA, pH 8 1 mmol/L Pefabloc SC 3 mmol/L $BaCl_2$ 1.5 mol/L Urea DF: 12	Full-length plasma-derived VWF	Residual VWF binding to collagen, chromogenic ELISA End-point
[a]FRET[30,31,52,77]	LLOQ: 1%-3% Intra-assay CV: NR Inter-assay CV: ≤6%	PNP diluted in ADAMTS13-inactivated plasma	5 mmol/L Bis-Tris, pH 6 1 mmol/L Pefabloc SC 25 mmol/L $CaCl_2$ 0.005% Tween-20 DF: 50	VWF73 substrate	Fluorescence change (ex 355 nm, em 450/460 nm) Kinetic
[a]FRET[30,46,48,49,50,78]	LLOQ: 3%-5% Intra-assay CV: 6% Inter-assay CV: <10%	PNP diluted in assay buffer	5 mmol/L Bis-Tris, pH 6 1 mmol/L Pefabloc SC 25 mmol/L $CaCl_2$ 0.005% Tween-20 DF: 30	VWF-73 substrate	Fluorescence change (ex 340 nm, em 450/465 nm) Kinetic
FRET[3]	LLOQ = 0.3% Intra-assay CV = 1.8% Inter-assay CV = 1.7%	Heparinized PNP (minimally diluted in assay buffer)	50 mmol/L HEPES, pH 7.4 150 mmol/L NaCl 10 mmol/L $CaCl_2$ 1 mg/L BSA 0.05% Tween-20 protease inhibitor cocktail and 1 mmol/L PMSF DF: 2	rVWF71 substrate	Fluorescence change (ex 638 nm, em 658 nm) Kinetic
FRET[48]	LLOQ = 5% Intra-assay CV = NR Inter-assay CV = 5%-18%	Commercial calibrant diluted in ADAMTS13-inactivated plasma	Undisclosed components DF:20	VWF86-ALEXA substrate	Fluorescence change (ex 485 nm, em 530 nm) Kinetic
Chromogenic activity ELISA[32,35,48,50]	LLOQ: 0.3%-3% Intra-assay CV: ≤5.4% Inter-assay CV: ≤8%	Commercial calibrant diluted in assay buffer	Undisclosed components DF:31	VWF73-GST substrate	Chromogenic ELISA format (anti-GST capture; HRP conjugated anti-N10 monoclonal Ab to detect substrate cleavage) End-point

Abbreviations: Ab, antibody; CV, coefficient of variation; DF, dilution factor; em, emission; ex, excitation; GST, glutathione S-transferase; HRP horseradish peroxidase; LLOQ, lower limit of quantitation; NR, not reported; PNP, pooled normal plasma; r, recombinant.

[a]Exact detail varies between laboratories.

slightly longer peptides, modified sequences or fluorophores.[31] The peptides may contain: tags to allow their attachment to microtitre plates (eg His-tag); attached proteins to allow measurement of intact or cleaved substrate (eg glutathione S-transferase); or have fluorophores (eg 2-(N-methylamino)benzoyl group) and quenchers (eg 2,4-dinitrophenyl group) attached adjacent to the cleavage site in the fluorescence resonance energy transfer (FRET)-based assays. In chromogenic activity ELISA methods, substrate cleavage may be detected by loss of signal (eg removal of GST), or the increased binding of monoclonal antibodies against a neo-epitope generated after substrate cleavage (eg anti-N10 antibody).[32] In FRET-based assays, an increased fluorescence signal occurs due to the loss of quenching when the peptide is cleaved and the quencher is no longer in close proximity to the fluorophore. The activity ELISA has multiple steps (incubation, washing, reagent addition, etc.) and many laboratories have microplate readers, whereas the FRET assays can be set-up with automatic reagent addition, incubation and measurement, minimizing hands-on time, although a fluorescence microplate reader is required.

An ADAMTS13 activity assay based on the VWF73 peptide and surface-enhanced laser desorption ionization time-of-flight

(SELDI-TOF) mass-spectrometry has also been reported.[33] Although highly sensitive, instrumentation requirements make this assay impractical for clinical diagnostic use in most hospital settings.

More recently, particle-based automated assays have been developed. An automated chemiluminescence assay utilizes a two-step immunoassay involving magnetic particles coated with GST-VWF73 peptide substrate and chemiluminescent detection based on an isoluminol labelled monoclonal antibody that reacts with the cleaved peptide. It is a rapid assay (33 minutes), with good sensitivity and precision, but only has a three point calibration curve. Although there were some discrepancies compared to FRET-VWF73 and a chromogenic activity ELISA, with some samples showing inter-assay disparity at normal and high activity, there was a good correlation between the assays and high agreement in classifying samples with ADAMTS13 levels below 10 IU/dL.[34-36] Due to the reaction principle, these assays are not affected by icterus, lipaemia or plasma turbidity. However, the sample size in two of these studies was small and further evaluations may be needed before their widespread acceptance and regional regulatory bodies may require additional verification or validation procedures.

The ionic strength, pH, divalent cation and chloride ion concentrations are critical in ADAMTS13 assays, and all buffers should be fresh in order to avoid pH changes and potential bacterial contamination. Alternatively, some buffers can be stored for extended periods lyophilized, or prepared as stock solutions (eg 10× stock buffers) especially if filtered with a 0.22 μmol/L membrane. Beside the pH and ionic environment, the use of denaturants and differences in sample dilution factor between assays might have an impact on the binding kinetics of ADAMTS13 and its autoantibodies, so that varying degrees of dissociation could occur in different assay methods.[37] Finally, some assays utilize heat inactivated normal plasma (treated at 56°C for 30 minutes to denature ADAMTS13) as a diluent, to help preserve the ADAMTS13 environment and allow a linear standard curve.

3.2 | Point of care (POC)/rapid tests

A rapid, semi-quantitative test for ADAMTS13 activity is now commercially available,[38] which utilizes a flow-through cartridge and an activity ELISA principle, being completed within 30 minutes, without the need for specialized instrumentation or personnel. It has four indicator points (zero, 10, 40 and 80 IU/dL) and in comparison with a chromogenic activity ELISA in 220 patients with suspected TMA, showed 88.7% sensitivity, 90.4% specificity and 96.2% negative predictive value. The method is limited by its subjective visual interpretation and potential for interference by lipids (which might block the device membrane), haemolysis and icterus. The test is suitable for use in a POC environment as a screening tool and for the negative exclusion of TTP. When decreased activity is detected, it should be confirmed by bioassay in an accredited laboratory.

3.3 | Agreement/disparity between activity assays

A number of studies[21,34,35,39-52] have investigated the precision and comparability of different ADAMTS13 activity assays, although many of these have involved small sample numbers, limiting their clinical applicability. In general, these studies show that the various activity assays differ in the lower limit of quantification and linear range, while there are clear differences for certain clinical sample types, dependent on the exact assay comparison. CBA and peptide substrate-based assays are certainly disparate for samples from patients with truncated or mutant forms of ADAMTS13 in their plasma, although this is not surprising due to differences in the substrate length. The degree of discrepancy may vary between different CBA methods. Differences have also been noted between methods using the same peptide substrate, even in severe ADAMTS13 deficiency (<10 IU/dL). Sample collection

TABLE 2 Potential sources of discrepancy between activity assays

Variable	Likely sources of discrepancy
Calibrant	Normal plasma or rADAMTS13
Diluent	Inactivated plasma, buffer, pH and ionic strength
Dilution factor	Immune complex dissociation (causes falsely increased activity)
Reagents	Denaturants may cause Immune complex dissociation[37] (causes falsely higher activity)
Substrate type	Peptide length and sequence (influences sensitivity to defects involving the distal ADAMTS13 domains)
Interfering substances	Plasma colour (icterus, haemolysis, etc.) may influence FRET assays (causing false lower activity), but causative substances are washed away in CBA and chromogenic assays[54,55]
Haemoglobin	Increased plasma haemoglobin can protect VWF from ADAMTS13 cleavage[3,57,58] (reduced activity), but may have less effect on peptide substrates (higher values)
Anti-ADAMTS13 specificity	Antibody population may affect ADAMTS13 interaction sites present on VWF (causing reduced activity), but not smaller peptide substrates (which may give higher values)
ADAMTS13 mutants and truncated forms	May affect ADAMTS13 interaction sites present on VWF (causing reduced activity), but not smaller peptide substrates (which may give higher values)

and handling differences may contribute to these changes as well as a variety of *in vivo* and *in vitro* factors (Table 2). The results of these studies emphasize the need for standardization and further assay development. When choosing which ADAMTS13 assay to set up, selection will depend on local circumstances (including frequency and number of samples, required turnaround time, budget, available equipment and expertize). It is clear that it is important to use the same ADAMTS13 assay in a given patient when managing treatment.

3.4 | Standardization and calibration

An International Standard (IS) plasma for ADAMTS13 is available (12/252; NIBSC, South Mimms, UK)[53] and is calibrated for activity and antigen. This should be used as the primary calibrant for commercial calibrants and local assay standards should be traceable to the IS, with results being reported as International Units. Irrespective of which anticoagulant, or if serum is used, the assay calibrant should be of the same type as the sample, for example use pooled citrated plasma standard for citrated samples.[3] It should be noted that the IS calibration is derived from lyophilized citrated plasma and so discrepancy may occur if the test plasma is prepared using a different anticoagulant. The IS has not been assessed with modified assays; for example minimally diluted plasma and varying ionic strength. In the absence of IS availability, results should be reported as percentage of pooled normal plasma. Some commercial calibrants are only suitable for use in the manufacturer's kit and may give false low or high levels when used in other assay techniques, due to additives and buffering. Care should be taken with the use of stabilizers (frequently used in lyophilization of plasma), to avoid pH changes and alteration of the ionic environment, which can have marked effects on ADAMTS13 function.

3.5 | Sensitivity

ADAMTS13 assay sensitivity can be attributed to several factors, including the substrate type, assay method, instrument, buffer conditions and volume of plasma. ADAMTS13 has maximal activity at low pH (pH 6) and low ionic strength (zero NaCl) as opposed to pH 7.4 and salt concentrations similar to those in plasma (150 mmol/L NaCl). Generally, VWF peptide-based assays are more sensitive than those using multimeric VWF digest analysis. The first generation FRET-based assay sensitivity was limited (~3%-5% of normal ADAMTS13 activity),[30] whereas more recent, optimized FRETS, chromogenic activity ELISA and microparticle-based assays have a limit of quantitation of 0.2-0.3 IU/dL (Table 1). Sensitivity at very low ADAMTS13 levels ≤5 IU/dL for FRET-based assays can be obscured by baseline autofluorescence, temperature fluctuations and photobleaching associated with fluorophores.

New FRET assays, utilizing a rVWF71 polypeptide corresponding to VWF Gln 1599-Arg1668, containing mutations N1610C and K1617R, as well as an N-terminal Gly, are more sensitive and appear to overcome the problems of plasma colour, icterus and haemolysis (see below) by utilizing fluorophores with different absorbance and emission wavelengths.[3,5]

3.6 | Specificity

In assays using full-length VWF, it must be high purity, containing all normal multimer sizes and free of ADAMTS13 contamination. This may be checked by performing assay blanks and by measuring ADAMTS13 antigen. A variety of peptide substrates derived from the VWF A2 domain can be used to assay ADAMTS13 activity[5]; their specificity can be checked by a negative control containing 10 mmol/L EDTA. When performing assays, the operator should be aware that many reagent and plasma additives (EDTA, protease inhibitors and surfactants) as well as certain therapeutic materials can potentially interfere with assay performance, especially the FRET-based assays.

Bilirubin levels >100 μmol/L (although rarely seen in acute TTP plasmas) can reduce ADAMTS13 activity in the FRETS VWF-73 assay due to fluorescence quenching at the emission wavelength (450 nm).[54] This results in reduced baseline fluorescence measurement and apparent ADAMTS13 reduction. The CBA and chromogenic peptide ELISA are not affected by bilirubin, which is removed in the washing stages. However, in vitro studies suggest that unconjugated bilirubin may have a direct, dose-dependent inhibitory effect on cleavage of both peptidyl and native VWF substrates by ADAMTS13.[55] The influence of bilirubin may sometimes be reduced by using higher sample dilution, but this is limited by ADAMTS13 activity level and the detection limit. Bilirubin oxidase has been employed[56] to avoid quenching, but the method has not been fully validated for use with clinical samples. Some samples (with no visible icterus) exhibit low baseline fluorescence in FRET assays, so it is likely that certain other substances or therapeutic agents may influence the assay.

Haemoglobin has been shown to bind specifically to the VWF A2 domain and prevent cleavage by ADAMTS13,[57,58] using both full-length VWF and the VWF A2 domain as substrates. The effect of haemoglobin on shorter VWF peptide substrates, such as those used in the FRETS and chromogenic assays, is unclear. However, 2 g/L Hb reduced ADAMTS13 activity by ~10% in a FRETS-rVWF71 assay,[3] which could have a significant impact on patient management.

3.7 | Antigen assays

Immunoblotting procedures and ELISA assays have been used for ADAMTS13 antigen measurement and the latter is the method of choice in the clinical diagnostic laboratory when an assay is indicated. There are a variety of commercial kits and numerous polyclonal and monoclonal antibodies available for assays. The capture and detection antibody selection is critical as monoclonal antibodies directed against the central domains of the protein may

detect truncated forms and full-length ADAMTS13, while antibodies directed against the C-terminus may underestimate functional ADAMTS13 molecules.

3.8 | Antibody and inhibitor assays

ADAMTS13 autoantibody assays demonstrate the presence of specific immunoglobulins and are detected in iTTP. Depending on the ADAMTS13 domain specificity of the immunoglobulins, they may or may not inhibit the proteolytic activity. Inhibitory antibodies can be demonstrated in mixing tests and Bethesda type assays. Non-neutralizing antibodies can influence ADAMTS13 survival in the circulation and are clinically relevant. Autoantibody assays, where available, are therefore preferred.

3.8.1 | Autoantibody assays

Western blotting, immunocapture and ELISA assays for determination of ADAMTS13 autoantibody levels have been described,[59,60] but ELISA methods for immunoglobulin (Ig) G class autoantibodies are most widely used in clinical laboratories. The calibration of these assays, their reporting units and cut-off values vary; the results may not be interchangeable.

3.8.2 | Inhibitor tests

Patient plasma is heated at 56°C for at least 30 minutes (to inactivate ADAMTS13 and other haemostasis proteins, while leaving IgG unaffected), centrifuged to remove any precipitated protein and doubling dilutions are incubated with untreated pooled normal plasma (PNP), as a source of ADAMTS13, for 1 hour at 37°C. A control comprising heat-treated normal control plasma or buffer and untreated PNP is incubated in parallel. After incubation, the residual ADAMTS13 activity is measured in the patient and control samples. If an IgG inhibitor is present in the patient sample, the ADAMTS13 activity in the test sample will be lower than the control. In this case, the incubated dilutions of heat-treated patient plasma are assayed to determine inhibitor potency in a Bethesda type assay. Some methods do not utilize heat treatment and are therefore potentially sensitive to other types of inhibitor (eg anti-ADAMTS13 IgM). Anti-ADAMTS13 IgG is thought to be the most clinically significant immunoglobulin class in acquired TTP and heating makes the mixing test more specific, but adds a technical step and prolongs the analytical time. Assays based on inhibition of rVWF71 are very sensitive to inhibitory antibodies but are not widely used.[3]

The effects of different incubation temperatures and different assays for measuring residual activity have recently been investigated,[23] showing a good correlation between FRET-based and CBA-based inhibitor assays, although the pre-incubation at 37°C causes a reduction of plasma ADAMTS13 activity when tested in the FRET-based assay. A flow-based assay for ADAMTS13 inhibitor assessment has also been described.[61]

The mixing test inhibitor methods are prone to errors because of plasma manipulation and dilution of patient plasma with PNP limits the sensitivity. Dilution could potentially alter the equilibrium between free and bound antibody. Bethesda type assays of ADAMTS13 inhibition may be problematic due to the variability in antibody epitope specificity, affinity and reaction kinetics between patients. Standardization and variability were improved when minimally diluted plasma and conditions close to physiologic were used. FRETS-rVWF71 was ~2.5-fold more sensitive for inhibitory autoantibodies than FRETS-VWF73 using recommended protocols.[3]

Sporadic, weak antibodies may sometimes be detected and may be absent when a fresh sample is subsequently obtained. When antibody concentrations are high, it may be difficult to determine an exact antibody level, as the dilution curve may be nonparallel with the calibration curve. This can arise for several reasons: in acquired TTP, there are often a variety of antibodies present, directed against epitopes on the spacer domain, the thrombospondin-like repeats and other domains; these may vary in avidity and concentration, so that each antibody type is more or less pronounced in its effect depending on the dilution used; the reaction kinetics may vary between antibodies with different specificities and between patients; sample dilution may cause antigen:antibody complex dissociation.

There is currently no IS or reference material for ADAMTS13 antibody and inhibitor assays, meaning that numerical results are difficult to compare between different assay types.

4 | VALIDATION OR VERIFICATION OF ASSAY PERFORMANCE

FRETS assays (which are often used as reference methods in major haemostasis laboratories) are mainly developed in-house and are not certified by regulatory bodies (in vitro diagnostic device registration (CE mark) in the European Union and US Food and Drug Administration). Commercial kits including the most widely used chromogenic activity ELISA method have regulatory body approval in certain countries. Where they do not, or if tests are modified (eg use of bilirubin oxidase), they must be considered as a laboratory-developed test (LDT). ICSH recognizes that there is no universal definition of an LDT and it is therefore the responsibility of the individual laboratory/institution to decide whether a test system constitutes a LDT and to implement a validation procedure meeting their local regulatory requirements. This might include assessment of linear range, sensitivity limits, comparability, and precision, or require more extensive investigation/documentation.[62] After the validation of assays, a verification phase should be carried out before clinical implementation, to ensure that the assay is consistently producing acceptable results. This may be performed by comparison with another laboratory and participation

in external quality assurance (EQA) schemes. The uncertainty of measurement (UOM) should be estimated by each laboratory to aid in the interpretation of borderline results. Long-term internal quality control (IQC) data are used at each IQC level, to determine the mean and standard deviation (SD), expressed with 95% confidence (±1.96 SD).[63] Thus, a sample with 0.20 IU/dL activity and SD 0.03 would give UOM ± 0.04 IU/dL, indicating a true value between 0.14 and 0.26 IU/dL.

4.1 | Internal quality control, external quality assessment

Some commercial kits provide normal and abnormal control samples, and these should be run with every batch of tests to provide IQC. If these are not available, or if additional IQC is required, local controls using aliquots of plasma samples that have previously been assigned a potency and an acceptable performance range can be included. Ideally, these should have been originally collected in the same way as test samples. Performance should be monitored over a range of potencies. Commercial control samples should only be used in the methods for which they were designed, unless formal validation is performed, as differences in the buffer environment may influence their performance.

The United Kingdom National External Quality Assessment Service, the ECAT Foundation, the College of American Pathologists and the North American Specialized Coagulation Laboratory Association, all provide external quality assessment studies for ADAMTS13 assays, with several proficiency testing surveys each year.

4.2 | Criteria for validation of each assay run

A calibration curve should be performed with each batch of tests to avoid reagent and analyser variability. Some activity assay methods may be particularly temperature sensitive. IQC values should be within their target range. Test results should be within the linear portion of the calibration curve; if they have levels above this range, they should be repeated at higher dilution; if below the range, they must be reported as less than the lower limit of quantification. In kinetic assays, the baseline readings should be checked and very low or high values may indicate an analytical problem.

4.3 | Consensus recommendations on assays for ADAMTS13, autoantibodies and inhibitors

- Functional FRET-based assays or chromogenic activity ELISA methods are recommended as front line assays as they are sensitive, show good precision and are simpler to use, being completed in a few hours.
- Rapid point of care assays may have utility as screening methods or "out of hours" emergency tests, but there is currently limited performance data.
- Every calibrator should be traceable to the International Standard Plasma (12/252) for assaying ADAMTS13 activity in citrated plasma samples.
- When reporting results: indicate the type of assay performed and use the correct units (eg IU/dL) for activity and antigen assays. If calibrants traceable to the IS are not available, use percentage of pooled normal plasma. State the reference range for the method.
- High and low activity controls should be included in each assay run. Do not use commercial controls in methods other than those intended for their use.
- Protocols should be validated after any modification.
- If gross icterus interferes in some FRET assay methods, the problem can sometimes be resolved by assaying at a higher dilution, treatment with bilirubin oxidase, or using a chromogenic activity ELISA. A comment regarding potential assay interference should be added to the laboratory report.
- If artefactual, in vitro haemolysis is likely (eg secondary to difficult venipuncture or use of a small gauge needle), fresh blood samples should be obtained. If this is not possible, a comment regarding potential assay interference should be added to the laboratory report.
- Where assay results do not match the clinical picture, or congenital TTP is suspected and ADAMTS13 activity results are normal or only show a mild reduction, collagen-binding assays should be considered.
- If decreased ADAMTS13 activity (<20 IU/dL) is detected in a new patient, an ADAMTS13 antibody assay or inhibitor test should be performed. If the results do not match the clinical picture, potential EDTA contamination should be considered and where possible, fresh blood samples obtained.
- Wherever possible, use the same ADAMTS13 assay when studying a patient longitudinally to manage treatment.

5 | CLINICAL UTILITY OF ASSAYS

Due to the rarity of TTP, ADAMTS13 assays are not widely performed and remain mainly confined to specialized laboratories. However, recent advances in the development of ADAMTS13 assays and recognized importance of ADAMTS13 testing in TMA differential diagnosis indicate that there is an urgent need for a timely and reliable ADAMTS13 result for the clinician. Clinical scoring systems such as the PLASMIC score[64] (platelets $<30 \times 10^9$/L, creatinine <176 μmol/L, international normalized ratio <1.5, mean corpuscular volume <90 fL, evidence of haemolysis, no cancer or organ transplantation) may help clinicians decide on the urgency of ADAMTS13 testing and the likelihood of a positive TTP diagnosis.

When a patient presents with a clinical scenario and blood film of suspected TMA (Fig. 1), deficiency of ADAMTS13 activity of <10 IU/dL

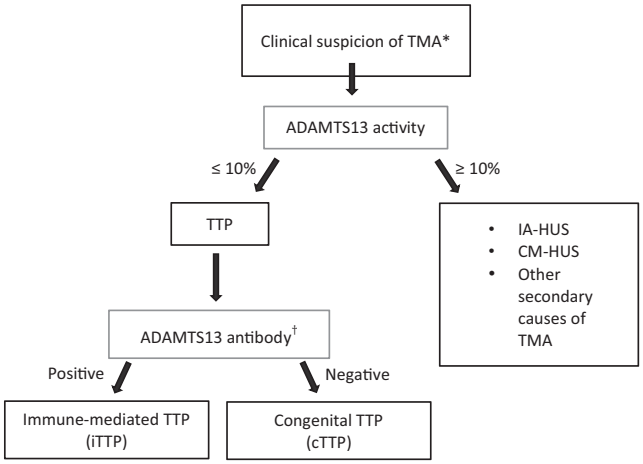

FIGURE 1 ADAMTS13 testing for the diagnosis of TTP and other TMA. *Based on clinical assessment including Clinical Gestalt, PLASMIC score, laboratory investigation (haemoglobin, platelet count, fragments, and creatinine level). †High titre ADAMTS13 antibody (ELISA) or functional inhibitor of ADAMTS13 activity measured by Bethesda assay. CM-HUS, complement mediated haemolytic uremic syndrome; IA-HUS, infection associated haemolytic uremic syndrome; TMA, thrombotic microangiopathy; TTP, thrombotic thrombocytopenic purpura

(and particularly <5 IU/dL) is diagnostic for TTP.[8] Levels greater than 10 IU/dL suggest another diagnosis depending on the clinical circumstances and include consideration of HUS (both Shiga toxin or aHUS) and secondary TMA.

The turnaround time for ADAMTS13 activity results varies considerably depending on local practice and logistic reasons, so often PEX and prednisolone treatment is initiated early while awaiting the assay result. However, PEX is less effective in TMA patients with ADAMTS13 >10 IU/dL who do not have TTP,[11] so timely access to an assay avoids the cost, inconvenience and side effects of this ongoing, unnecessary intervention. In addition, access to new TMA therapies such as caplacizumab (a nanobody that blocks VWF binding to platelet glycoprotein Ib)[65] and rituximab (an immunosuppressant anti-CD20 antibody)[66,67] for acquired TTP and eculizumab (a C5 complement inhibitor) for CM-HUS[68] is further motivation for rapid, widely available screening of ADAMTS-13 activity. This could be achieved with recently reported automated chemiluminescent immunoassay[34-36] or rapid POC tests.[38] Subsequent confirmation of an abnormal result with an ADAMTS13 FRET or activity ELISA assay may be required until these newer, rapid assays are further validated. Ideally, the first sample should be taken prior to initiation of plasma-based therapy to avoid interference of donor plasma ADAMTS13 or dilution of the anti-ADAMTS13 autoantibody. However, in a series of 18 acquired TTP patients followed throughout daily PEX therapy, >75% still showed <10 IU/dL ADAMTS13 activity immediately before the fourth exchange procedure.[69]

Differentiating between cTTP and iTTP relies upon the detection or absence of an ADAMTS13 autoantibody or inhibitor.[13,70] The determination of a clinically significant positive IgG antibody varies between laboratories depending on the assay and reagents used and the limit of normality varies both numerically and in the units used. If no significant ADAMTS13 antibody is found, hereditary deficiency of ADAMTS13 is suspected and replacement therapy with plasma or ADAMTS13 containing FVIII concentrates may be considered rather than immunosuppression.[70] In these cases, *ADAMTS13* genetic testing is helpful to confirm the diagnosis of cTTP.[71] ADAMTS13 phenotypic and genetic testing of the proband's parents and siblings is suggested to further confirm the hereditary nature of TTP, especially for compound heterozygous mutations and for genetic counselling.

Precision of the ADAMTS13 activity assay around the 10 IU/dL cut-off level and clinical interpretation of the result is still problematic, leading to a wide variation in clinical decisions and management. An indeterminate range of between 10 and 20 IU/dL activity necessitates repeat assay, and it is more likely to be significant for TTP diagnosis if a high level of ADAMTS13 autoantibody is also detected. Interpreting the clinical significance of an isolated ADAMTS13 antibody result is challenging because of the limited data defining abnormal levels and arbitrary normal cut-off values depending on the laboratory, leading to the misdiagnoses of a detectable but not pathological ADAMTS13 antibody. Furthermore, standardization of the method and nomenclature of the ADAMTS13 Bethesda assay are lacking. Up to 25% of iTTP patients have autoantibodies that do not neutralize the ADAMTS13 activity, but they are thought to be pathological by removal of ADAMTS13 through an immune complex or other clearance mechanism.[72]

Around 30%-50% of iTTP patients have exacerbation (falling platelet count to below the normal range, an increased LDH level and the need to restart PEX within 30 days of the last exchange) or relapse (falling platelet count below the normal range, with or without clinical symptoms, more than 30 days after stopping PEX and requiring therapy) with the risk of exacerbation or relapse related to persisting ADAMTS13 deficiency of <10 IU/dL and detectable autoantibodies at the end of plasma exchange despite a normal platelet count, or during disease remission, respectively.[60,70,73,74]

Frontline rituximab in acute iTTP and pre-emptive rituximab in iTTP patients in remission with persisting low ADAMTS13 activity have been considered, but its use, particularly in patients with a first event is debated as not all iTTP patients experiencing a first event will relapse and not all iTTP patients who have a low ADAMTS13 activity during remission will relapse. Caplacizumab, which should ideally be started in the early phase of the acute TTP event for maximum benefit, was also shown to prevent exacerbation in iTTP patients achieving clinical response, but still presenting severely reduced ADAMTS13 activity, supporting its extended use until the underlying autoimmune disease is resolved. Thus, the appropriate use of both rituximab and caplacizumab in iTTP demands the availability of accurate and timely ADAMTS13 testing.

Mortality is also increased in iTTP patients with the highest IgG ADAMTS13 antibody and lowest ADAMTS 13 antigen level,[75] perhaps suggesting increased clearance or immune complex binding of

circulating ADAMTS13 antigen and these patients may especially benefit from new therapies. ADAMTS13 antigen levels are not routinely measured and have little clinical utility in the absence of activity assays[24]; further work is required to understand the clinical significance of this finding.

It is apparent there is an urgent need for standardizing ADAMTS13 testing for the different clinical situations of the new TMA diagnosis, whether it is iTTP, cTTP, CM-HUS, TTP monitoring or prediction of TTP relapse.

5.1 | Consensus recommendations on clinical utility of assays

- ADAMTS13 activity <10 IU/dL is diagnostic for TTP in patients presenting with a clinical scenario and blood film consistent with TMA.
- An alternative diagnosis and repeat ADAMTS13 assay should be considered in TMA patients presenting with an indeterminate range of 10-20 IU/dL ADAMTS13 activity.
- Alternative diagnosis/therapy should be considered in patients with an ADAMTS13 activity >10 IU/dL, especially when >20 IU/dL, as they rarely respond to PEX.
- The overall clinical picture/treatment of the patient should always be reviewed with close interaction with clinicians. This is important to avoid unnecessary tests and to ensure that appropriate tests meet clinical needs, especially when non-TTP expert clinicians request assays. Clinical scoring systems such as the PLASMIC score may assist in providing guidance for the necessity of ADAMTS13 testing.
- ADAMTS13 antibody assays should be used to decide whether the patient has cTTP or iTTP; definite cTTP diagnosis requires *ADAMTS13* genetic testing.
- Nomenclature, normal range, reporting units and methodology (eg inhibitory and noninhibitory antibodies) need further standardization.
- Follow-up ADAMTS13 activity testing is important as persistently low levels increase the risk of exacerbation and TTP relapse.
- ADAMTS13 antigen levels are not clinically useful in the absence of activity assays, but may prove helpful in predicting mortality risk in iTTP patients with high IgG ADAMTS13 antibody level, although further study is needed.

6 | CONCLUSION

Our knowledge concerning ADAMTS13 assays has advanced considerably in recent years, but there are still considerable knowledge gaps concerning: the impact of all pre-analytical variables, calibration of autoantibody assays, the place of epitope-specific assays, and which assays to use in different clinical scenarios.

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CONFLICT OF INTEREST

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